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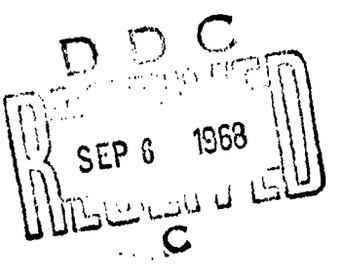

ATTEMPT TO GROW ENCEPHALITOZOON IN TISSUE CULTURE

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Since the Encephalitozoon was found by Wright and Craighead (1922), to be the causative organism of a spontaneous paralytic disease in young tamed rabbits, many investigators have reported the presence of the natural infection to be wide-spread among various mammals and birds. Furthermore, the presence of the natural infection among small experimental animals has been reported by Levaditi (1924), Cowdry and Nicholson (1924), Perrin (1943) and Tazaki (1956) etc., such as in the mouse, rat, guinea pig, and sparrow, etc., and also the incidence of infection has been investigated. The fact that the natural infection of Encephalitozoon is present among various experimental animals would seriously interfere with the study, experiment, and preservation of strains of Encephalitozoon, and would at times lead to a difficulty in evaluating the experimental results. Therefore, it is thought to be a very significant step to obtain the artificial tissue culture of Encephalitozoon for further study on Encephalitozoon in order to avoid the pre-existing natural infection among animals. A result of the experiment is reported.

Material and Method

Encephalitozoon was isolated from a rat and maintained in the mouse peritoneal cavity through passage every two and half weeks to supply for the experiment. Three kinds of tissue were used in the experiment.

1. Culture by He. La. Cells

He. La. cells used here were a strain maintained through passage at the Department of Obstetrics and Gynecology, School of Medicine, Keio University. Culture medium consisted of 48% Hanks solution, 50% human serum, and 2% chick embryo juice mixed with 100 u/cc of Penicillin and

100 /cc of Streptomycin. The pH of the solution was always maintained at 7.3-7.8 and deviation of pH was constantly corrected to the above for the optimal growth of He. La. cells. Culture of He. La. cells and their growth were obtained in 1cc of the culture medium placed in a small culture tube with a small rectangular cover glass as a supporting body within, after incubation at 37°C. for three days. Ascites obtained under sterile technique from Encephalitozoon infected mouse was then added few drops into the tube and again incubated. Thereafter, the medium was replaced every five days, considering the growth condition and the falling-off of He. La. cells and propagation of Encephalitozoon, until the 25th day when the rectangular cover glass was taken out and examined under a microscope after staining by Giemsa Stain to evaluate the propagation of Encephalitozoon and parasitic existence of the organism in He. La. cells.

2. Method by Brain Tissue Culture

The brain of chick embryo at the 14th or 15th day after incubation was resected out under sterile technique and this was made into four pieces of one cubic millimeter in size, after washing in Gay's solution. The larger blood vessels and meninges were removed under sterile technique. The pieces of brain were then placed in a solution consisting of one drop of serum from human cord blood, one drop of plasma from young male chicken blood which was heparinized and two drops of 50% solution of the supernatant from crashed one week old chick embryo after centrifugation for 30 min. at 2000 r/min., then mixed together in a large cover glass. Then a few drops of ascites of the Encephalitozoon infected mouse were added and a concave glass was placed and the slide was sealed with paraffin. As soon as the medium in the slide coagulated, the slide was turned over and placed in an incubator at 37°C. Microscopic examination was made daily on this slide thereafter for ten days, beyond which time the tissue would die because of vital changes in the culture medium, in order to evaluate the growth of brain cells especially glia cells and behavior of Encephalitozoon.

3. Culture in Hen's Eggs

Ascites was first collected from Encephalitozoon infected mice. Then 0.1cc of this was innoculated separately on chorioallantoic membrane into the allantoic cavity, into the amniotic cavity, and into the omphalic sac of live hen eggs in incubation for one week. The innoculation method was that the incubated eggs were inspected for the presence of a growing chick embryo and a triangular window was made on the egg shell where there were no blood vessels to expose the shell membrane. Then a pin point opening was made on the shell membrane with care so as not to injure the underlying chorioallantoic membrane. After the two membranes were separated by collapsing the air chamber through a hole on the shell, the chorioallantoic membrane was exposed by excising the shell membrane carefully, in case of innoculation on the membrane. A few drops of the prepared ascites were then put on the membrane and the shell was sealed with cello-tape. The egg was again placed in an incubator for another two weeks and an observation

was made on the innoculated chorioallantoic membrane which was excised from the egg and smeared on slides, and then Giemsa stained for microscopic examination as well as the macroscopic examination of the alteration of the membrane. Innoculation into the allantoic cavity was made on eggs after one week's incubation. A small hole was made on the shell near the air chamber and after sterilizing the area with alcohol, 0.1cc of the infected ascites was injected with a syringe through the chorioallantoic membrane. After re-incubation for two weeks, the allantoic fluid was collected and smeared on slides for the examination of the organism. In the case of innoculation into the amniotic cavity, a square window was made with a file in the egg shell on the top of air chamber and the underlying shell membrane was excised and the inner shell membrane at the bottom of the air chamber was also peeled off from the chorioallantoic membrane with care so as not to injure the blood vessels. A syringe was then inserted through the chorioallantoic membrane directly toward the chick embryo until the contact was felt with the amniotic membrane on the tip of the needle. Then a small air bubble, which was contained in the syringe on purpose, was first rejected in order to ascertain that the tip of the needle was located exactly within the amniotic cavity and 0.1cc of the ascites was injected. The hole in the shell was sealed with cello-tape and after the reincubation for another two weeks, a microscopic examination was made on the amniotic fluid. Innoculation into the omphalic sac was made through a hole made in the shell on the top of air chamber, with a 3 cm syringe inserted into the sac after penetrating through both the inner shell membrane and the chorioallantoic membrane. The content of the omphalic sac was examined under a microscope following the two-week incubation.

In addition, each site of innoculation was made into the emulsions with sterile physiologic saline solution of ten times in volume and each of them was then innoculated into the peritoneal cavity of mice individually, and their ascites were examined for the presence of the organism after two and half weeks. Furthermore, five eggs each from four different innoculated groups were brought through to be hatched and the brain and liver of the chicks were prepared into the emulsion with a physiologic saline solution of ten times in volume. The emulsions were then innoculated into the peritoneal cavities of mice, two mice for each of four different methods of innoculation (Mouse A and Mouse B), in order to determine the establishment of Encephalitozoon infection in the chicks. When the infection was recognized both in Mouse A and Mouse B, the experiment was said to be definitely positive.

Results

1. Culture by He. La. Cells

In culture by He. La cells, Encephalitozoon existed for three to four days within the macrophage cells which were throught to be from ascites of infected mice, but no increase was seen in the macrophage cells and the Encephalitozoon also showed no splitting or propagation. He. La. cells

showed no influence by the addition of the organism at all and grew as well as the contrast of the pure culture. Microscopic examination of the Giemsa stained preparation in this series was carried out every other day for a period of twenty-five days but no evidence of Encephalitozoon infection in He. La. cells was seen.

2. Method by Brain Tissue Culture

Observation in this experiment was possible only for the period of ten days because of this particular culture method by hanging drop technique. Macrophage cells containing Encephalitozoon were recognized until the 5th day, but no evidence of infection or propagation of Encephalitozoon was seen within the brain cells or even actively propagating glia cells. Also, no changes were noted in these cells by the presence of Encephalitozoon.

3. Culture in Incubated Hen Eggs

Microscopic examination of the Giemsa stained preparations from each site of innoculation after a total incubation period of three weeks revealed no organisms in the allantoic cavity, amniotic cavity and omphalic sac, but there were four our of twenty cases from innoculation on the chorio-allantoic membrane. Macroscopic examination, however, revealed no changes such as thickening or clouding of the membrane. The experiment, in which the emulsions of each of the innoculated sites with physiologic saline solution were separately passed into the peritoneal cavity of mice, revealed the organism in two out of twenty cases of chorioallantoic innoculation. One of these two positive cases also showed the organism in the smear of the membrane before the passage into the mouse periotneal cavity but the other was negative by the smear. Innoculation either into the allantoic cavity, amnoitic cavity, or omphalic sac yielded no organism either in the smear or in the passage into mice. In the experiment, in which the emulsions of brain and liver from completely hatched chicks were separately innoculated into the peritoneal cavity of mice, four groups out of six groups (each group consisting of two mice) were positive in the case of innoculation on the chorioallantoic membrane and one group had one positive mouse but the other was negative. No organism was found in mice after the innoculation of either brain tissue or liver from the other sites of innoculation in hen eggs. Even in the case of innoculation on the chorioallantoic membrane, the passage of the chick brain emulsion did not yield any organism. The growth of Encephalitozoon on the chorioallantoic membrane, however, was noted to be poorer than that of the peritoneal cavity of mice.

Consideration and Summary

The affinity of Encephalitozoon to the various cells artificially cultured in this series of experiments was found to be very poor, so that the propagation and maintenance through generations of Encephalitozoon without using the experimental animals are considered to be very difficult

at the present time. It is thought that Encephalitozoon possesses less motility and less growth speed than Toxoplasma, and is not capable of invading He. La. cells, whereas Toxoplasma invaded and grew upon the cells as previously reported by Arai (1958). There seems to be certain conditions provided in the part of incubated hen egg for the growth of Encephalitozoon but the growth is far from satisfactory as compared to that of Toxoplasma in incubated egg culture as reported by Warren and Russ (1948). The fact that Encephalitozoon does not grow well in various tissue cultures, unlike Toxoplasma which is a very similar organism, with the exception of its abundant growth in macrophage cells in the peritoneal cavity of mice, may be explained on the basis of its affinity which is very poor towards these cultured tissues used in this series of experiments. If artificial culture of the macrophage cells of mice peritonea is successful, the tissue culture of Encephalitozoon can be also made successfully, but the culture of the macrophage is very difficult at the present time and can not be maintained long enough to supply the series of experiments. In the case of innoculation on the chorioallantoic membrane in this experiment, the growth of the Encephalitozoon was seen at the site of innoculation and also occasionally in the chick embryo, whereas a similar innoculation experiment with Toxoplasma revealed the presence of infection not only on the chorioallantoic membrane but also in all of the rest such as allantoic cavity, amniotic cavity, omphalic sac and embryo. The fact that the emulsion of a hatched chick liver yielded the organism indicates, however, that a certain degree of invasive infection is taking place. In this series of experiments, no direct alteration or changes of the cultured tissues were noted at all by the innoculation of Encephalitozoon, even in the case of positive infection on the chorioallantoic membrane. In all cases of fully hatched chicks that were positively infected during the incubation by innoculation on the chorioallantoic membrane and their liver emulsions yielded the organism after passage into mouse peritoneal cavity, no changes or differences at all were noted in their appearance and in sectioning of the organs. The fact that the cells of the central nervous system, especially glia cells, which are often affected in the case of natural infection in animals, did not show any influence by Encephalitozoon in the experiment may indicate that there would be certain cells with specific affinity to the organism in the central nervous system.

Conculsion

A series of experiments were made to investigate the infection and growth of Encephalitozoon in He. La. cells, young chick brain cells, and incubated hen eggs.

1. Encephalitozoon was cultured together with He. La. cells but no infection or growth of the organism was seen within the He. La. cells.

2. Encephalitozoon remained alive for several days within the macrophage cells of mouse peritonea in the culture medium of He. La. cells.

3. No significant changes were noted in He. La. cells by addition of Encephalitozoon.

4. No evidence of invasion of Encephalitozoon was seen in the chick brain cells that were cultured on slides together with the organism.

5. The growth of chick brain cells was not interfered by innoculation of Encephalitozoon.

6. Encephalitozoon existed for several days within the macrophage cells of mouse peritonea in the culture medium of the brain cells.

7. Innoculation of Encephalitozoon on chorioallantoic membrane of hen eggs under incubation yielded the organism in four out of twenty cases as the end of two weeks.

8. Intraperitoneal innoculation of the emulsion, prepared from the infected chorioallantoic membrane by innoculating Encephalitozoon on the membrane of incubated eggs, into the peritoneal cavity of mice yielded the organism in two out of twenty cases.

9. Intraperitoneal innoculation of the liver emulsion, prepared from fully hatched chicks that were innoculated during the incubation by Encephalitozoon on chorioallantoic membrane, into the peritoneal cavity of mice yielded the organism in four out of six groups fully.

10. Innoculation of Encephalitozoon into the allantoic cavity, amniotic cavity, and omphalic sac of incubated hen eggs did not yield any organism at the end of two weeks and also no organism was found from the fully hatched chicks.

11. No cases of death of incubated eggs innoculated by Encephalitozoon were seen and also no abnormalities were seen in hatched chicks.

12. The various methods applied ~~here~~ in author's experiment are not capable of growing and maintaining Encephalitozoon properly.

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Tables

Table 1

Inoculation Sites	Chorioallantoic membrane		Allantoic cavity		Amniotic cavity		Omphalic cavity	
Method of Determination	Smear	Passage into mice	Smear	Passage into mice	Smear	Passage into mice	Smear	Passage into mice
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	+	-	-	-	-	-	-	-
4	-	+	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	+	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	+	+	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	+	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-
16	-	-						
17	-	-						
18	-	-						
19	-	-						
20	-	-						

Recovery of the organism from various sites of innoculation.

Table 2

Material used for passage	Brain		Liver	
Mouse	A	B	A	B
1	-	-	+	+
2	-	-	+	+
3	-	-	+	-
4	-	-	-	-
5	-	-	+	+
6	-	-	+	+

Organ passage experiments from hatched chicks innoculated on chorioallantoic membrane during incubation.

Table 3

Material used for passage	Brain		Liver	
Mouse	A	B	A	B
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-

Organ passage experiments from hatched chicks innoculated into allantoic cavity during incubation.

Table 4

Material used for passage	Brain		Liver	
Mouse	A	B	A	B
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-

Organ passage experiment from hatched chicks innoculated into amniotic cavity during incubation.

Table 5

Material used for passage	Brain		Liver	
Mouse	A	B	A	B
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-

Organ passage experiments from hatched chicks innoculated into omphalic sac during incubation.